IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Victor Raso
Application No.:	09/992,994
Filing Date:	November 6, 2001
Title:	IMMUNOLOGICAL CONTROL OF β-AMYLOID LEVELS *IN VIVO*
Art Unit:	1652
Examiner:	Patterson, C.

DECLARATION OF CHRISTINE BOURGEOIS

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Christine Bourgeois, do hereby declare and say:

I. From before December 2, 1997 it was my understanding that Vic Raso was attempting to obtain funding to support himself, his family, and his β-amyloid vaccine research

1. From 1996 to 2001, I worked as a technician in the laboratory of Vic Raso at the Boston Biomedical Research Institute (BBRI). Vic was a Senior Scientist at the BBRI.

2. According to Vic and other colleagues, I was told that he ran out of funding around 1995. Through my colleagues, I became aware that Vic cut his own salary to pay my salary and medical benefits.

3. To get funding, Vic told me that he was actively working on numerous different grant applications.

4. In early 1997 Vic told me that he had not worked in the Alzheimer's field before. This was one reason why Vic's grant applications were being rejected.

5. During this time, Vic was constantly at his computer while I would conduct experiments in the lab. When we would have conversations he would say that he was working on grant applications that all concerned Alzheimer's Disease, particularly the use of antibodies to β-amyloid. Vic informed me that our goal was to develop a vaccine to treat Alzheimer's Disease using these antibodies.

6. I was not aware of the details, but through discussions with other colleagues it was mentioned that Vic was trying to save his career at BBRI so that he could conduct his research. He worked many nights and weekends writing the grant applications and obtaining the data to be included in them. I would also help assist in the proofreading of those grants before he submitted them for review. I do not recall the details of those grants, but know that it was in regards to our Alzheimer's research. During weekdays he spent most of his time writing grants while I took care of daily laboratory routines and carried out experiments according to his instructions, and in the evenings and during weekends he spent most of his time working in the lab. Vic worked diligently and would always still be there when I would leave in the evening. When I would return to the lab on Monday mornings there were always remnants from experiments performed by Vic from over the weekend. I would then organize my day according to what experiments had been conducted over the weekend.

II. Our experimental work during this time was devoted to an Alzheimer's vaccine

7. Grant applications require data. Accordingly, Vic designed and I conducted many experiments to support these grant applications. Because Vic was focused on grant-writing and not on publishing, he told me that the focus of those experiments was primarily to obtain data needed for the grant applications.

8. Some of the experiments conducted in support of the grant applications are summarized in the attached chart. [Ex. A]. The laboratory notebook pages and printouts containing the data from our experiments are also attached. [Ex. B]. I believe these documents are readily understood by a scientist in the field. However, I provide the following explanation of the experiments I performed for further clarification.

9. Our experiments encompassed active as well as passive vaccination for the treatment and/or prevention of Alzheimer's disease. Work on these basic concepts was simultaneously initiated sometime early in 1997 when we synthesized the first β-amyloid peptides. We proceeded to use the β-amyloid peptides as an active vaccine in normal and transgenic mice. We subsequently derived monoclonal antibodies from immunized mice and utilized those anti-β-amyloid antibodies as passive vaccines.

A. Materials and Methods

1. Monoclonal Antibody Production

10. A key aspect of Vic's research involved monoclonal antibodies. It was my understanding that a significant proportion of time and effort was devoted to formulating strategies for new antibodies and antigens, generating numerous lines of monoclonal antibodies, assaying for their specific activities, isolating the lines of interest, and measuring the quality and quantity of the individual line.

11. Obtaining a high quality monoclonal antibody of interest was a time-consuming process each time. A mouse was immunized by injection of an antigen X to stimulate the production of antibodies targeted against X. The antibody forming cells were then isolated from the mouse's spleen. Monoclonal antibodies were produced by fusing single antibody-forming cells to tumor cells grown in culture. The resulting cell is called a hybridoma. Each hybridoma produces relatively large quantities of identical antibody molecules. By allowing the hybridoma to multiply in culture, it was possible to produce a population of cells, each of which produces identical antibody molecules. These antibodies are called "monoclonal antibodies" because they are produced by the identical offspring of a single, cloned antibody producing cell. The production of hybridoma generally involved the following steps:

1. Immunization of mice
2. Spleen removal and preparation of a single cell suspension
3. Myeloma cell preparation
4. Fusion of spleen cells and myeloma cells
5. Post-fusion cells cultured in hybridoma selection medium (HAT)

6. Collection and dispersion of peritoneal macrophages

7. Addition of fused cells to microtiter plates with macrophages post fusion (this was done the same day as the fusion). This experiment usually yielded 8-12 96-well microtiter plates.

8. Culture of cells: 37°C, 5% CO2 [fed with HAT medium]

9. 7 to-21 days post fusion: observe and numerate hybridoma clones

10. Screen for specific antibody production

11. Expand cultures positive by screening test

12. Reclone by a limiting dilution technique all positive hybridoma clones to assure monoclonality and to select for the fastest growing cell line with the greatest antibody production. Hybridomas would be recloned periodically (after 3-4 months of culture) to prevent overgrowth of your preferred culture by mutants or cells expressing an altered phenotype.

13. Inject recloned hybridoma cells into BALB/c mice which had received an i.p injection of 0.3 ml of pristane 7 days previously. Collect ascites 7-21 days later.

14. Guard against loss of hybridoma by storing several amps of each clone in liquid nitrogen.

12. Because these time-consuming steps were routinely carried out, no specific entries were recorded in a laboratory notebook on a daily basis. Some records are available which noted the final phase of screening of hybridoma lines that presented "promising" characteristics, and experimental data showing the results of the ELISA assays identifying these lines, and eventually leading to the successful isolation of the monoclonal antibodies that exhibited potentially therapeutic activities for

Alzheimer's disease, as claimed in the invention of the patent application at issue. Therefore, it should be inherently appreciated that considerable amount of time had been spent on the earlier phase of the antibody production, despite the fact that few entries were recorded (as being routine procedures).

13. The attached documents document the production of numerous monoclonal antibodies. [Exs. A and B]. The above-step necessarily occurred each time. Otherwise, the monoclonal antibody could not have been created.

2. Cell Culture

14. Besides the hybridomas used to create monoclonal antibodies, several lines of cells grown in culture were used in the experiments we conducted. Maintaining cell cultures involves some tasks on a daily basis, such as changing culture medium for each dish of cells, preparing and maintaining growth media and other solutions and reagents needed for culturing cells, and monitoring the growth state of the cells. In addition, for the propagation of cell lines, other components of maintenance are involved.

15. We used several different types of cultured cells for different purposes. The time required to maintain cells in culture was between less than an hour up to several hours per day. Most cell lines grown in culture require maintenance and routine care approximately every 2-3 days. When an experiment needed to be started from a frozen stock of cells, a vial of cryo-preserved cells were thawed and placed in an appropriate growth medium. Cells were then allowed to proliferate until they reach a desired confluence (density or fullness per given growth surface, e.g., a culture dish).

Depending on the growth rate of the particular cell type, this could take up to one week or sometimes longer before we were able to use the plate of cells for an experiment.

3. Animal Protocols

16. During the course of our work on the present invention, we had to learn a number of new methods and techniques. It was my understanding that Vic was also researching the latest developments in the field because he had me obtain research articles from the Treadwell Library at Massachusetts General Hospital on various lab techniques or other topics related to his research. Among those techniques, we needed to familiarize ourselves with general animal handling procedures, namely, the breeding of mice and the maintenance of the mouse colonies. In particular, the initial phase of establishing a colony of mice was challenging. New animals were generally placed in quarantine for several weeks in order to minimize potential cross-infection. Upon being deemed free of contaminants, the animals were then allowed into the main facility. Before any experiments could be performed using the mice, we had to establish a new colony from the initial set of mice.

17. Each of our animal experiments required many subjects in order to compensate for individual variations and errors. To accommodate the requirement, we expanded a colony to sufficient size which took lots of my time. Because the gestation period for mice is approximately 18-20 days, it would take, even under ideal circumstances, almost three weeks to obtain the first set of litters. Upon arrival of a litter, each mouse would be tagged, then "genotyped" (i.e., genetically determined by

DNA analysis) to confirm its genetic profile. The results of this process would simply be recorded by me in a notebook as "positive (+)" or "negative (-)."

18. For genotyping mice, I used PCR-based analyses. Every mouse was genotyped to ensure genetic characteristics of interest. This involved collecting tissue samples from each animal (from ear tissue), preparing genomic DNA from the tissue sample by digesting the tissue and extracting DNA, then using the resulting DNA to carry out genomic PCR. Subsequently, I ran DNA gels to analyze the PCR samples to identify the genotype of each animal in the colony.

19. Our first attempts to expand a mouse colony stumbled initially, likely due to our inexperience with the procedures. Additionally, we found that these mice would often cannibalize one another. Nevertheless, eventually we managed to obtain a colony of the mice. Generally, mice reach maturity at the age of approximately 5-7 weeks. At this point, they could be used for certain experiments; alternatively, they could be used for breeding.

20. Another challenge we discovered in animal experimentation is to obtain enough mice that are "age-matched" "background-matched" and "sex-matched", in order to obtain consistency in data acquisition. In particular, some of our experiments using animal models involved "aged" (~1+ year old) mice in which the age-dependent manifestation of Alzheimer-like disease was mimicked. To perform these experiments, it naturally required one year or longer after the arrival of a litter. For these reasons, it often takes a relatively large colony of mice to sustain a much smaller number of animals actually used in experiments.

21. In addition, our experiments involved a series of immunization (injections) of mice. Typically, a first injection of antigen to trigger immune response ("immunization") was given to an animal; subsequently, about four to six weeks later a second immunization (called "boost") is given. The right interval between these injections is important in ensuring effective immune response in the mouse. Therefore, would take a minimum of approximately two months from the time of the initial injection before we could obtain an animal that exhibited a robust immune response to a given β-amyloid antigen.

22. The attached documents document the production and/or maintenance of the mouse colony. [Exs. A and B]. The above-steps necessarily occurred each time. Otherwise, the colony could not have been created or maintained.

23. Similarly, the attached documents document the immunization of mice. [Exs. A and B]. The above-steps necessarily occurred each time. Otherwise, the immune responses could not have been generated and boosted.

B. Our β-amyloid Alzheimer's vaccine experiments

24. Before December 2, 1997, Vic had conceived of β-amyloid active and passive Alzheimer's vaccines, and we had generated a number of hybridoma cell lines in an effort to establish useful monoclonal antibodies to β-amyloid ("Alz PS"). We had obtained ELISA assay results, showing some positive and negative results for the Alz PS clones. [Ex. C (B001450)]. We continued to screen for monoclonal antibodies, and we obtained ELISA sheet-testing a control and a 6E2 ascites on an Alz PS coated plate versus an Alz1-43 coated plate. [Ex. C (B001451)].

25. We had successfully used those monoclonal antibodies in an antibody:polyethylene glycol (PEG) assay that measured the binding of native, human full-length radioactive (^{125}I) β-amyloid to monoclonal anti-phenylstatine antibodies 5A11, 2E3, 5G11, 2H11, 11H2. [Ex. C (B001452-B001453)].

26. In particular, I recall that the 5A11 antibody showed very promising results, which was very exciting.

27. Human β-amyloid exists in several forms and, as a native protein, may not be expected to be antigenic. Thus, we had synthesized a mixture of four peptides encompassing amino acids 10-25 of native human β-amyloid plus a cysteine for thioether coupling to an antigenic carrier. These peptides included the native human sequence plus three modified versions in which the two phenylalanine residues were randomly substituted with a statine transition state analog (abbreviated PS or sta).

28. I ran spectral analyses to make sure correct fragments were generated. More specifically, the presence of each of those designated peptides was verified by mass-spec analysis.

29. The HPLC (high pressure liquid chromatography) analysis, which I carried out frequently, yielded peaks representing synthesized peptides of discrete masses, and the data also indicated that the material in the late peak did not dissolve well in water and may have been an amyloid complex. In addition to the HPLC analysis, mass spectral analyses were also carried out in order to verify the identity of the peptides. [Ex. C (B001467-B001481)].

30. We then went on to immunize four mice with the Alzheimer's peptides. Initially, the mice were vaccinated with 50 micrograms of Alz-TS-KLH in complete

Freund's adjuvant (CFA) and then boosted with the same antigen in incomplete Freund's adjuvant (IFA) four months later. [Ex. C (B001482)]. The timing of the second boost is important for ensuring effective immune response in order to optimize the subsequent step of monoclonal antibody production.

31. Hybridoma cell fusions for the production of monoclonal antibodies were created a few weeks after the vaccination with IA. This procedure involved isolating the spleen cells from immunized mice and carrying out "fusing" of the immune cells with highly proliferative lines of cells in culture. At this point, we grew these hybridomas in culture and diluted the samples to isolate clonal colonies. Each colony, representing dividing cells derived from a single cell origin (clonal), was manually isolated with the use of small device termed cloning rings, then the isolated colony was in turn grown separately to establish each hybridoma line. [Ex. C (B001483-B001493)]. This process typically takes several weeks to a few months.

32. We also worked on establishing peptides to be used as a vaccine. Assorted human β-amyloid peptides were synthesized, which were distinct from those made previously but were constructed using a similar random substitution strategy. This peptide mixture was abbreviated AlzSta as opposed to the Alz-phenylstatine or AlzPS designation for the first peptide. The human amyloid β-peptide termed Alz 14-25 C-Y was designed both as a vaccine to generate antibodies and as a central region peptide that was labeled with radioactive iodine. [Ex. C (B001494-B001519)].

33. In order to convert these peptides into usable vaccines, we then carried out chemical coupling of the peptides with an immune stimulant that acts as a carrier. The coupling of the Alz-statine peptide mixture synthesized to both maleimide

activated KLH and OVA, which are commonly used carriers in the conjugation of peptides for antibody production. This process created antigens for both vaccination and for ELISA analysis of monoclonal antibodies. [Ex. C (B001520)].

34. To generate an ascites which is a rich source of these different monoclonal antibodies directed against human β amyloid, immunization of mice using these antigen was carried out. Five mice were vaccinated with the AlzStaKLH peptides in CFA. The mice were then boosted with the same antigens in IFA five weeks later. [Ex. C (B001521-B001523)].

35. We analyzed monoclonal antibodies generated using the β-amyloid peptide vaccine Alz-TS-KLH. We screened antibodies by ELISA assays. The spleen for the hybridoma fusion was obtained from one of the mice initially immunized. As predicted, this ELISA data proved that vaccination with the composite vaccine produced some antibodies that bound to native, human, full-length, β-amyloid, some that bound exclusively to the phenylstatine transition state peptide, and some that cross-reacted with both the native and transition state peptides. [Ex. C (B001524-B001556)].

36. Vic carried out comparative analyses of the composite vaccine. Vic demonstrated that immunization of an animal with a human β-amyloid vaccine can effect the distribution of human β-amyloid in the body of an animal. [Ex. C (B001557-B001558)]. Thus, it was shown that β-amyloid vaccination had the potential for a positive therapeutic impact on the Alzheimer's disease process. I was present when these results were obtained; Vic showed me the data and explained to me the significance of the results.

37. We therefore started preparing frozen stocks (liquid nitrogen) of early hybridoma clones that produced Alzheimer's disease-related antibodies directed against human β-amyloid, including Alz phenyl Statine clones, which were derived from animals that had been immunized with the Alz-Phenylstatine vaccine. Similarly, frozen samples from the Alz-Statine clones that were derived from animals that had been immunized with the Alz-Sta peptide vaccine were also prepared. [Ex. C (B001559-B001584)].

38. Subsequently, we measured the direct binding of native, human, full-length, radioactive β-amyloid ("125I Alz1-45") to a vast array of monoclonal anti-phenylstatine antibodies. These antibodies were obtained from a hybridoma fusion that used the spleen of a mouse that was immunized with the first vaccine synthesized. [Ex. C (B001586-B001608)].

39. We began preparing a new set of hybridomas from the spleen of an animal immunized with the AlzStaKLH composite peptide vaccine. [Ex. C (B001609-B001663)]. Subsequent ELISA analyses followed.

40. We anticipated the need for an animal model to test the effectiveness of this form of therapy. Vic had read that such a model was freely available to researchers (Hsiao, K., P. Chapman, S. Nilsen, C. Eckman, Y. Harigaya, S. Younkin, F. Yang, and G. Cole. 1996. Correlative memory deficits, AB elevation, and amyloid plaques in transgenic mice. Science 274:99-102). Vic contacted a researcher who had a good animal model to make an arrangement for us to obtain such animals. After some time, he told me that he received a response from Dr. Hsiao to the request that

he had previously made for obtaining Alzheimer's animal model, which was called Tg2576.

41. Our animal facility at BBRI finally received the delivery of the requested transgenic mice, Tg2576. It was my understanding that we needed about 7 months to establish a viable Tg2576 colony of sufficient size to proceed with vaccination experiments. [Ex. C (B001734)].

42. We subsequently laid out a schedule for and began passive immunization of Tg2576 mice using the 5A11 monoclonal antibody. This antibody binds to human β-amyloid and was produced from a hybridoma fusion that used the spleen of a mouse that was immunized with the first vaccine synthesized. [Ex. C (B001800)].

43. The cited documents show that all the above experiments were performed before December 2, 1997. As can be seen from the attached chart [Ex. A] and the documents it cites [Ex. B], we continued to work in the lab on a β-amyloid Alzheimer's vaccine. For example, in Cage ID AA-92, the white and black mice were immunized with Alz-KLH in CFA on September 12, 1998 and boosted on October 24, 1998 while the brown mouse got KLH control immunizations on September 13, 1998 and October 24, 1998. These experiments were designed to test the injection of Alzheimer' s vaccines (active immunization) for therapeutic effect. The comments column reading DOA 9/16/97 2Bk=5A11 and the other which reads "DOA 9/16/97 Br=5A11" designate animals that were treated with the purified 5A11 monoclonal antibody. These kinds of experiments were designed to test the injection of Alzheimer's antibodies (passive immunization) for therapeutic effect. The 5A11

antibody binds human β-amyloid and was obtained from the hybridoma fusion which used one of the mice initially immunized. [Ex. C (B001760-B001799)].

44. On September 12, 1998, we outlined an immunization schedule and vaccine components, and for immunizing the ALZ Tg2576 mice. As indicated the vaccine was comprised of a mixture of different human native β amyloid peptide-KLH antigens. [Ex. C (B001804-B001812)].

The above statements are made with the knowledge that willful false statements may be punishable as provided by 35 U.S.C. 25 and 18 U.S.C. 1001.

Christine Bourgeois